

**Homo- and heterocellular junctions in cell cultures:
an electrophysiological and morphological study**

(Jonctions homo- et hétérocellulaires dans des cultures de cellules:
une étude électrophysiologique et morphologique)

THÈSE

présentée à la Faculté de médecine de l'Université de Genève
pour obtenir le grade de docteur en médecine

par

Alan Cedric Ross HYDE

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Thèse No 3145

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La Faculté de médecine, sur le préavis du professeur *Jean Posternak*, autorise l'impression de la présente thèse, sans prétendre par là émettre d'opinion sur les propositions qui y sont énoncées.

Genève, le 6 mars 1969

Le doyen:
Professeur WILLIAM GEISENDORF

Thèse No 3145



Ce travail a paru sous les noms des auteurs
A. HYDE, B. BLONDEL, A. MATTER, J.-P. CHENEVAL, B. FILLOUX et L. GIRARDIER
dans K. AKERT AND P. G. WASER (Eds.), *Mechanisms of Synaptic Transmission*
(*Progress in Brain Research*, Vol. 31), Elsevier, Amsterdam, 1969, pp. 283-311.

A mes parents

A ma femme

A mon fils

Je prie le professeur J. POSTERNAK, Directeur de l'institut de physiologie de la Faculté de Médecine de l'Université de Genève, d'accepter mes remerciements pour les précieux conseils et encouragements qu'il m'a prodigués tout au long de ce travail.

Veuille le Dr. L. GIRARDIER, professeur assistant à l'institut de physiologie de la Faculté de Médecine de l'Université de Genève, inspirateur et animateur de ce travail, trouver ici l'expression de ma profonde gratitude.

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Je remercie enfin Mlle. B. FILLOUX et M. M. RÜPHI de leur assistance technique aussi souriante qu'efficace.

Homo- and Heterocellular Junctions in Cell Cultures: An Electrophysiological and Morphological Study*

A. HYDE

INTRODUCTION

In the preceding contribution to this symposium, Weidmann has extensively discussed the problem of electrotonic coupling between cells of the cardiac Purkinje fibres and between cells of the myocardium proper. For both these tissues, there seems to remain little doubt that low-resistance intercellular junctions subserve the function of cell-to-cell propagation of excitation. Weidmann also gave some examples of other tissues in which evidence of electrotonic junctions has been found, and in which the role of electrotonic coupling does not seem to lie in the conduction of electrical signals. One can reasonably assume that, if electrotonic coupling is due to the presence of low-resistance connections between cells, then it will probably be associated with some degree of metabolic interaction between the interconnected cells, since the latter's ionic and micromolecular concentrations will tend to equalize across the junction. It seems fairly well established that the intracellular concentrations and movements of cations are fundamental to organic metabolism and cell permeability control, at least in certain tissues (Mosinger and Kujalova, 1966; Ho and Jeanrenaud, 1967; Letarte and Renold, 1967; Clausen *et al.*, 1968). In this context, it would seem highly interesting to study the properties of heterocellular junctions exhibiting electrotonic coupling.

Preparations containing such junctions can easily be obtained by culturing trypsin-dispersed heart cells. The cultures contain a mixture of cardiac myoblasts and fibroblasts in various proportions. Electrotonic coupling is regularly found between myoblast and myoblast, as was to be expected, and also, although to a lesser degree, between myoblast and fibroblast, and between fibroblast and fibroblast. Thus, cultures of cardiac cells offer the opportunity of investigating the properties of three functionally distinct types of junction.

A purely morphological comparison of myoblast-myoblast (M-M) junctions on the one hand, and of myoblast-fibroblast (M-F) and fibroblast-fibroblast (F-F) junctions on the other hand, suggested that the tighter electrotonic coupling found in the former was merely due to the lower resistance of the junctions between myo-

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blasts. Indeed, large areas of tight apposition of membranes (nexuses) are readily found at M-M junctions, whilst at M-F and F-F junctions, only locally restricted contacts of the membranes are seen. However, a detailed analysis of the electrophysiological data showed that the situation was not quite so simple, and that the greater effectiveness of the M-M coupling was to a considerable extent due to the higher specific resistance of non-junctional membrane in these cells.

Furthermore, cultures containing a mixture of fibro- and myoblasts suggest that the metabolism of a cell can effectively be modified by the presence of low-resistance junctions connecting it to a neighbouring cell of a different type, since the membrane potential of fibroblasts is no less than 3 times higher in a culture containing myoblasts than in a pure fibroblast culture.

MATERIALS AND METHODS

(a) Cultures

(1) Culturing technique

The culturing technique was adapted from those described by earlier investigators (Fänge *et al.*, 1957; Crill *et al.*, 1959; Harary and Farley, 1963; Lehmkuhl and Sperelakis, 1963; Mark and Strasser, 1966). The hearts of approximately a dozen 2- to 7-day-old Charles River rats of both sexes were excised as aseptically as possible, and minced together down to fragments 0.5–1 mm in diameter. The fragments were washed for two consecutive periods of 5 min in sterile phosphate buffer solution (PBS) (Dulbecco and Vogt, 1954) with gentle magnetic stirring. The PBS was then sucked off and discarded, and the fragments were resuspended in 10 ml of pre-warmed trypsin solution (0.25% trypsin in Ca^{2+} - and Mg^{2+} -free buffered saline). The vessel was placed in an incubator at 37° over a magnetic stirrer and left there for 10 min, during which time dissociation of cells took place. After 10 min the vessel was taken out of the incubator, the fragments were allowed to settle on the bottom, and the cloudy supernatant, containing a mixture of isolated fibro- and myoblasts, was gently sucked off, and either placed in a centrifuge tube with 5 ml cold culture medium, or discarded. The fragments of heart tissue were subjected to 8 such periods of trypsin digestion, and the resulting fractions were numbered in chronological order. The fractions retained for culturing were then centrifuged at approximately 1300 g for 5 min. The supernatant was discarded, and the pellets were resuspended in culture medium by gently sucking them up and down a large-bore pipette, pooled, and distributed in 6-ml aliquots into sterile polystyrene Petri dishes.

12 hearts usually provided an adequate inoculum for 6 dishes. The cultures were incubated at 37° with automatic pH control (pH maintained at 7.4 ± 0.05 by CO_2 in air against 7.1 mM HCO_3^-). 24 h later, the cultures were washed with warm PBS, in order to remove cells which had not become attached to the dish, and given 6 ml of fresh medium. Thereafter, medium renewals took place regularly at 48-h intervals.

The culture medium used in these experiments was Eagle's minimum essential medium (M.E.M. Powder Medium, Gibco) supplemented with 0.3 w/v lactalbumin

hydrolysate + yeast extract (Difco Laboratories) and 10% v/v calf serum. As mentioned above, the pH was buffered by 7.1 mM NaHCO_3 against CO_2 in air. The osmolality of the complete medium, as determined by the freezing point method, was 270 mOsm/kg, and its ionic composition was as follows.

Cations (mequiv./l)		Anions (mequiv./l)	
Na^+	148	Cl^-	127.21
K^+	5.36	HPO_4^{2-}	0.95
Ca^{2+}	0.90	HCO_3^-	7.1
Mg^{2+}	0.48	Organic (amino acids etc.)	19.48

(2) Purification technique

Selection of the trypsinization fractions which were to be cultured or discarded was determined by the cell type desired. Fraction 1 was always discarded, since it mostly contained cellular debris.

Mixed cultures. When mixed cultures, containing at the outset approximately equal proportions of fibro- and myoblasts, were desired, all fractions except the first were retained for culturing.

Myoblast-enriched cultures. Predominantly myoblastic cultures were obtained by taking the hearts of 2-day-old rats, and by discarding the first 3 trypsinization fractions, which mostly contained fibroblasts. In addition, advantage was taken of the fact that fibroblasts became attached to the plastic dishes within 2–3 h after being placed in the incubator, while myoblasts required a longer period. The highest degree of myoblast enrichment was reached by decanting the cell suspension into fresh dishes after about 3 h in the incubator, since by that time most of the fibroblasts had firmly adhered to the initial dish, whereas the myoblasts were still floating free in the medium. In the most successful experiments, the degree of purity of myoblast cultures exceeded 95% for the first 3 days. However, mitotic activity is much higher in fibroblasts than in myoblasts (Mark and Strasser, 1966), so that after 5–6 days of incubation the degree of purity fell to about 50%. Hence the need for very large inocula, if one wishes a myoblast culture to attain confluence before the inevitable fibroblast contaminants have pervaded the culture to an intolerable degree. A more detailed account of the purification technique will appear elsewhere (Blondel *et al.*, 1969).

Fibroblast-enriched cultures. When predominantly fibroblastic cultures were desired, 6–7-day-old rats were selected, and only fractions 2 and 3 were cultured. After 3 h in the incubator, the medium, together with the unattached myoblasts, was discarded, and replaced by fresh medium.

(b) Intracellular recordings

The recording system consisted of 2 conventional micropipettes filled with 3 M KCl (15–30 M Ω resistance) mounted on 2 separate micromanipulators. Each microelectrode was connected *via* an Ag–AgCl wire to the central terminal of an independently

mounted 3-terminal mercury switch, of which the 2 remaining terminals were connected respectively to a stimulus isolation unit, and to the input of a preamplifier with input capacity neutralization, an input resistance greater than $10^{12} \Omega$, and a grid current smaller than 10^{-13} A (M. J. Richez, Electronics Laboratory of the Faculty of Medicine, Geneva). With this arrangement, each microelectrode could be used alternatively to record transmembrane potential or to deliver current pulses, while at all times remaining intracellular. It was often possible to leave one of the microelectrodes in the same cell for over an hour without depolarizing it. The extracellular fluid was earthed through the 50Ω output resistance of a calibration unit.

Square current pulses were delivered to the preparation through a stimulus isolation unit, a $10^8 \Omega$ series resistance to ensure current constancy, and the input of a current monitor. The absence of interelectrode coupling was ascertained by routine checking.

The cells were observed through a phase-contrast microscope, with a total magnification of $500\times$. This proved sufficient to exclude confusion between fibroblasts and myoblasts, even when the latter happened to be quiescent.

(c) Experimental procedure

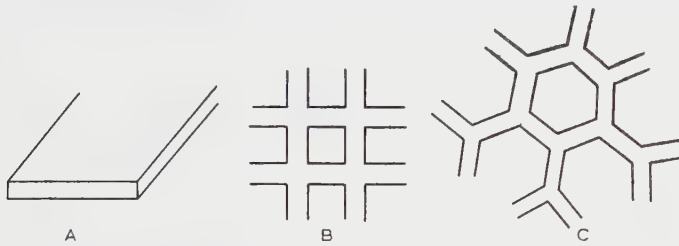
All impalements were carried out on confluent cultures in culture medium, at $30\text{--}32^\circ$.

At the outset, the mercury switches were in the "record" position, and remained so until both electrodes recorded almost identical resting and action potential amplitudes. One of the switches was then flipped into the "current" position, and hyper- or depolarizing pulses of current (5–20 nA, 120 msec duration) were passed through the corresponding microelectrode, while the other recorded the potential change across the membrane of the other impaled cell, some distance away. After a photograph of the electrotonic potential had been taken, the situation was reversed, and another record was taken. Finally, both microelectrodes were switched to the "record" position, and a control photograph was taken, in order to ascertain that neither cell had depolarized during the procedure. Records of impalements during which one or both cells depolarized by more than 5 mV were discarded. The distance between the two microelectrode tips was estimated with an eyepiece graticule. One of the microelectrodes was then withdrawn and inserted into another cell, and the whole procedure was repeated as many times as necessary to explore the field under observation. The interelectrode distances ranged from 3 to 400μ . Only photographs on which the current pulse terminated just before the upstroke of an action potential were analyzed. When enough points had been obtained, the culture was fixed, dehydrated, and embedded according to the technique described by Matter *et al.* (1969). Semi-thin sections were cut perpendicular to the plane of the culture, and photographed through a phase-contrast microscope, in order to estimate the average thickness of the culture, which was based on at least 30 measurements at randomly selected points on the culture.

THEORY

A model had to be developed to permit interpretation of the measured input resistance in terms of the specific membrane resistance and of the lumped specific resistance of the intracellular medium. A sheet of cultured cells should be reasonably well described by a two-dimensional model, and the external medium can be made of negligible resistance by leaving a 3-mm layer of liquid over the cells, thus fulfilling two simplifying assumptions often made in describing electrotonic spread in heart muscle. The major difficulty arises in defining the geometry of the connections between cells, since identification of low-resistance junctions is impossible to ascertain morphologically as well as topographically without the help of the electron microscope.

Fortunately, the geometrical factor turns out to be of secondary importance. Four geometrically different models have been described (Woodbury and Crill, 1961; George, 1961; Tanaka and Sasaki, 1966), and all four lead to equations of the same form for the distribution of electrotonic potential as a function of the distance between the current electrode and the recording electrode.



Scheme 1. A, 'Sandwich' model, Woodbury and Crill (1961). B, Rectangular lattice model, Tanaka and Sasaki (1966). C, Bifurcating lattice model, George (1961).

In steady state conditions, each of these models can be described by the differential equation

$$\frac{d^2 V(r)}{dr^2} + \frac{1}{r} \cdot \frac{dV(r)}{dr} - \frac{1}{\lambda^2} \cdot V(r) = 0 \quad (1)$$

where r is the distance between the current electrode (at $r = 0$) and the recording electrode, $V(r)$ is the potential at distance r , and λ the attenuation length, a constant which has to be defined for each model. The derivation of eqn. (1) for the "sandwich" model is given in the appendix (p. 306). The definition of λ in this case is

$$\lambda^2 = \frac{R_l \cdot d}{R_i}$$

where R_l = leakage resistance of a cm^2 of culture (Ωcm^2)

R_i = specific resistance of intracellular medium, lumped for myoplasm and intercellular connections (Ωcm)

d = thickness of the sheet of cells (cm)

The suitable solution of eqn. (1) is a modified Bessel function (eqn. 11 in the Ap-

pendix). By a simple curve-fitting procedure described in the Appendix, the values of R_i and R_e can be estimated from the measured electrotonic spread of potential.

RESULTS

(a) Contractile behaviour

In mixed and myoblast-enriched cultures, spontaneous, rhythmic beating was always observed 24 h after culturing. At first, as reported by others (Harary and Farley, 1963; Lehmkuhl and Sperelakis, 1963; Mark and Strasser, 1966), individual cells or cell clusters beat at independent frequencies, but within 2–3 days in our conditions, as the cultures tended towards complete confluence, contractions became synchronous throughout. Complete confluence was achieved between 3 and 6 days after culturing, depending upon the density of viable cells in the initial inoculum. By the time mixed cultures had attained complete confluence, they contained approximately equal proportions of both cell types. The myoblasts tended to assemble in roughly circular clusters, whereas the fibroblasts filled in the empty spaces between the myoblast communities. In general each cluster of myoblasts was in contact with neighbouring clusters through one or more strands of myoblasts, and the whole myoblast population of the culture contracted simultaneously. Occasionally a cluster was found completely surrounded by fibroblasts. These isolated clusters also contracted synchronously with the remainder of the myoblast population. It was observed in young, mixed cultures, which had not yet reached complete confluence, that a pair of myoblasts which did not appear to touch each other, and which did not make contact with other myoblasts, beat synchronously provided a fibroblast was interposed between them. This finding is similar to the observations of Mark and Strasser (1966). At all temperatures between 37 and 30°, the frequency of spontaneous contractions was lower in mixed cultures than in “pure” myoblast cultures. This may be because mixed cultures were usually obtained from slightly older rats than “pure” myoblast cultures.

Occasionally, a solitary group of 2–3 myoblasts was found in a fibroblast-enriched culture. In that event, the myoblasts were either completely quiescent, or showed weak, sluggish contractions.

(b) Electrophysiology

(1) Myoblasts in “pure” cultures

A series of records obtained from a successful impalement of 2 myoblasts belonging to an almost pure myoblast culture (fibroblast contaminants estimated at less than 5%) is shown in Fig. 1. As can be seen on the first and last photographs of the series, the resting potentials of both cells were practically identical throughout the impalement. Resting potential values ranged from -55 to -75 mV (mean 63 ± 9.5 mV S.D.). The membrane potential during “diastole” was usually flat, but occasionally showed slow diastolic depolarization, as well as the rounded upstrokes of action

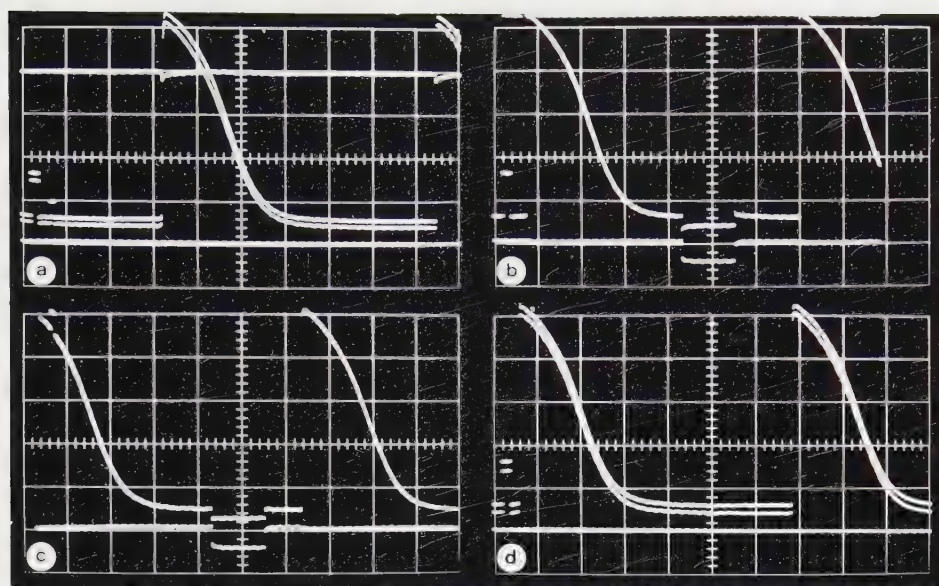


Fig. 1. Example of a set of transmembrane potential records obtained from a pair of cultured myoblasts in a pure culture. The interelectrode distance was $50\ \mu$. Frames a and d show the quasi-equality of the membrane potentials of both cells, before and after current had been passed respectively. Frames b and c show the electrotonic potentials when a square pulse of hyperpolarizing current (9 nA, 120 msec, bottom trace) was passed in succession through the two microelectrodes. Potential calibration pulse in all frames: 20 mV, 20 msec. O potential was one major division below the upper border of the graticule.

potentials typically associated with pacemaker activity. Pacemaker activity in cultured heart cells has already been reported by several authors (Lehmkuhl and Sperelakis, 1963; Crill *et al.*, 1959; Fänge *et al.*, 1957). In any given experiment, no more than 2–3 different cell clusters could be explored. Under these conditions it was observed that if one myoblast in a cluster displayed pacemaker activity, then the whole cluster showed the same behaviour. On the other hand, in most cultures it was difficult to locate the pacemaker even when as many as 50 impalements were made. Slow diastolic depolarization without rounded upstrokes of action potentials, indicative of driven cells with latent pacemaker potentiality, were also occasionally observed. The average resting potential of non-pacemaker cells was 62 mV ($n = 20$), that of pacemaker cells was 59 mV ($n = 38$), and that of latent pacemaker cells 59 mV ($n = 20$). The presence of a short plateau after the peak of the action potential was regularly observed in cultures derived from the hearts of very young rats (2–3 days old), whilst no plateau was noted in cultures of older hearts (6–8 days post natum). This finding is in broad agreement with the data of Bernard and Gargouil (1963) and Bernard *et al.* (1968), who reported the presence of a plateau preceding repolarization in foetal rat heart and its disappearance after birth. As illustrated by Fig. 1, the amplitude of the membrane potential deflection due to the current pulse was usually identical to within a few tenths of a millivolt, whether the current was delivered through electrode 1 or electrode 2. Several voltage–current plots (Fig. 2) have shown

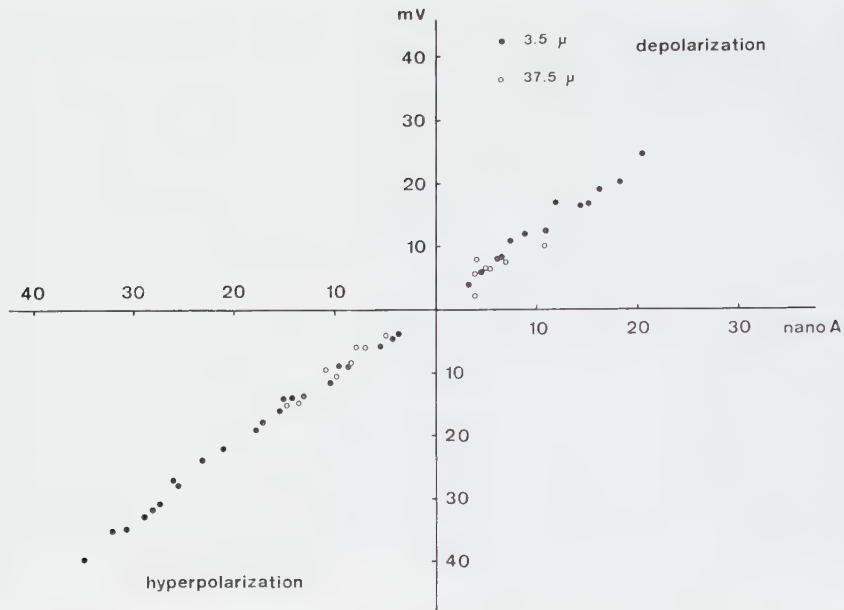


Fig. 2. Superimposed current-voltage plots obtained from two pairs of cultured myoblasts. Black circles, interelectrode distance 3.5μ ; both microelectrodes appeared to be in the same cell; pure culture. Open circles, interelectrode distance 37.5μ ; at least one cell junction was interposed between the electrodes; mixed culture.

that the junctions between cardiac myoblasts do not rectify to any appreciable degree within a considerable range of membrane potentials.

In all experiments, the transmembrane potential of one impaled cell was electronically subtracted from that of the other cell, and the subtraction trace displayed on the oscilloscope screen, enabling the observer to determine which of the two cells fired first. In this connection, it is of interest to point out that in "pure" myoblast cultures, as well as in mixed cultures, the firing sequence of a pair of impaled myoblasts was usually fixed throughout the entire observation period. Occasionally, however, an impalement of a pair of myoblasts showed variation of the firing sequence. Sometimes the switch of priority took place abruptly from one action potential to the next, as illustrated in Fig. 3, whilst in others it took place gradually, over several beats.

(2) *Myoblasts in mixed cultures*

The frequency with which pacemaker cells were impaled was not significantly different in mixed cultures from that encountered in "pure" myoblast cultures. The average resting potential of myoblasts in a mixed culture tended to be somewhat lower (mean 50 ± 10.3 mV S.D.) than in "pure" cultures.

Impalements in which one electrode was inserted into a myoblast, and the other into a fibroblast, showed that there was no fixed rule as to the sequence of firing: sometimes the myoblast fired first, at other times the action potential appeared first in the fibroblast.

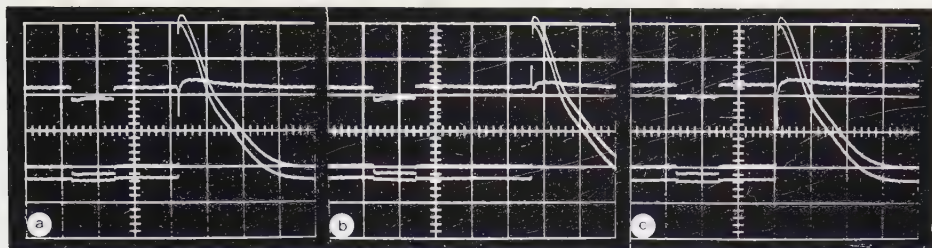


Fig. 3. Example of an abrupt switchover of the firing sequence of two myoblasts in a mixed culture. Three consecutive action potentials. The two bottom traces are the membrane-potential recordings, and the upper trace is the subtraction of one membrane potential from the other. The sharp spikes on the subtraction trace provide an indirect measurement of the delay between the upstrokes of the action potentials of both cells. Note the variation in the amplitude of these deflections from one action potential to the next, indicative of a variation in the firing delay, and the reversal of the firing sequence in the middle frame. Sweep speed 0.1 sec/division. Voltage calibration 20 mV per major graticule division.

(3) Myoblasts in fibroblast-enriched cultures

Impalements of solitary myoblast contaminants, occasionally found in fibroblast-enriched cultures, yielded resting potentials of the same magnitude as those of the surrounding fibroblasts. Low-amplitude (5–10 mV) upward deflections, synchronous with the sluggish contractions mentioned above, when these were present, were also observed. These deflections were also displayed by the fibroblasts in the immediate vicinity of the myoblast islets.

(4) Fibroblasts in "pure" cultures

Fibroblasts in "pure" cultures (less than 1% myoblasts) proved much more difficult to impale successfully than myoblasts. The membrane of fibroblasts seemed to tear with much greater ease than that of myoblasts. As a rule the membrane potential of fibroblasts in "pure" cultures showed a sharp transient, 35–40 mV in amplitude, at the moment of penetration, after which it stabilized at a value between –10 and –20 mV.

(5) Fibroblasts in mixed cultures

A striking difference was found between the membrane potential of fibroblasts in mixed cultures, as compared with "pure" fibroblast cultures. As stated above, the membrane potential of fibroblasts in "pure" fibroblast cultures usually stabilized at values between –10 and –20 mV. In contrast, fibroblasts belonging to a mixed culture exhibited resting potentials identical in amplitude to those of the surrounding myoblasts, *i.e.* –40 to –60 mV, as well as barely attenuated action potentials. Provided the criterion of a satisfactory double impalement was satisfied, *i.e.* quasi-equality of resting potentials of both cells, the total amplitude of the action potentials of a given fibroblast was roughly equal to that of the myoblast impaled in its neighbourhood.

A feature common to all double impalements, regardless of the nature of the cells impaled, was that the membrane potential of the first cell to be successfully penetrated

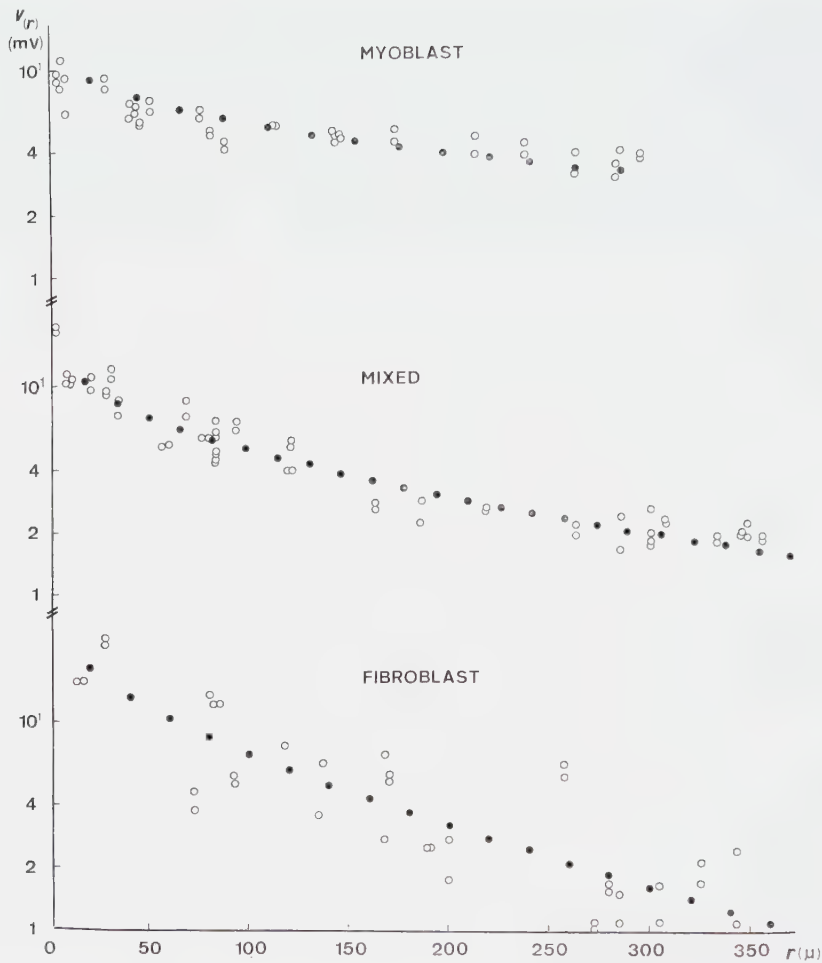


Fig. 4. From top to bottom: three semilogarithmic plots of the electrotonic potential against the interelectrode distance, obtained respectively from a pure myoblast, a mixed, and a pure fibroblast culture. The potential values were linearly normalized for $I_0 = 10^{-8}$ A. Open circles, experimental points. Black circles, best-fitting Bessel function, from which the attenuation lengths were obtained. The values of the latter were 1100μ for the myoblast culture, 400μ for the mixed culture, and 200μ for the fibroblast culture.

regularly fell by a few millivolts at the moment the second cell was punctured. As sealing in of the second microelectrode proceeded, both membrane potentials gradually rose to their stabilization level. This phenomenon was also observed when one of the impaled cells was a myoblast and the other a fibroblast. The amplitude of the transient depolarization of the first-impaled cell was more markedly dependent on the interelectrode distance in fibroblast cultures than in myoblast cultures. This is in accord with the shorter attenuation length found in fibroblast cultures.

Typical examples of potential *versus* distance plots for a "pure" myoblast, a "pure" fibroblast, and a mixed culture, are shown in Fig. 4, in which the logarithmic ordinate

TABLE I
SUMMARIZED ELECTROPHYSIOLOGICAL RESULTS

λ = attenuation length, nV_o = constant determined by the boundary conditions, r_i = effective resistance of intracellular phase, d = thickness of culture, Age = age of culture, R_i = lumped specific resistance of intracellular phase, R_l = leakage resistance of a cm^2 of culture, R_{i1} and R_{l1} were computed on the assumption that leakage of current occurred through the upper membranes only, *i.e.* by halving the leakage current given by eqn. 12 (Appendix).

Predominant cell type	λ (μ)	nV_o (mV) $\cdot 10^3$	r_i ($M\Omega$)	d (μ)	Age (days)	R_{i1} (Ωcm)	R_{l1} (Ωcm^2)	R_{i2} (Ωcm)	R_{l2} (Ωcm^2)
Myoblast	1100	2.19	1.39	2.06	4	286	16820	350	20691
Myoblast	1100	2.04	1.39	1.19	2	165	16820	202	20570
Myoblast	1100	1.59	0.98	3.92	3	384	11860	415	12826
Myoblast	1000	1.60	0.90	4.00	4	360	9000	388	9700
Myoblast	1000	1.60	0.90	3.95	3	356	9000	383	9700
Mixed	850	1.59	1.01	4.62	6	467	7297	517	8092
Myoblast	800	3.82	2.35	2.25	2	529	15000	574	16320
Myoblast	780	2.55	1.62	3.55	2	575	9856	621	10464
Mixed	700	1.66	1.02	—	4	—	5000	—	5439
Mixed	400	3.18	2.03	3.03	4	615	3250	691	3648
Fibroblast	250	12.10	7.31	—	6	—	4563	—	5968
Fibroblast	200	7.64	4.61	2.87	3	1325	1844	1556	2168
Fibroblast	180	11.46	7.06	6.78	4	4790	2287	6712	3208
Fibroblast	80	25.48	16.00	—	5	—	1021	—	3560

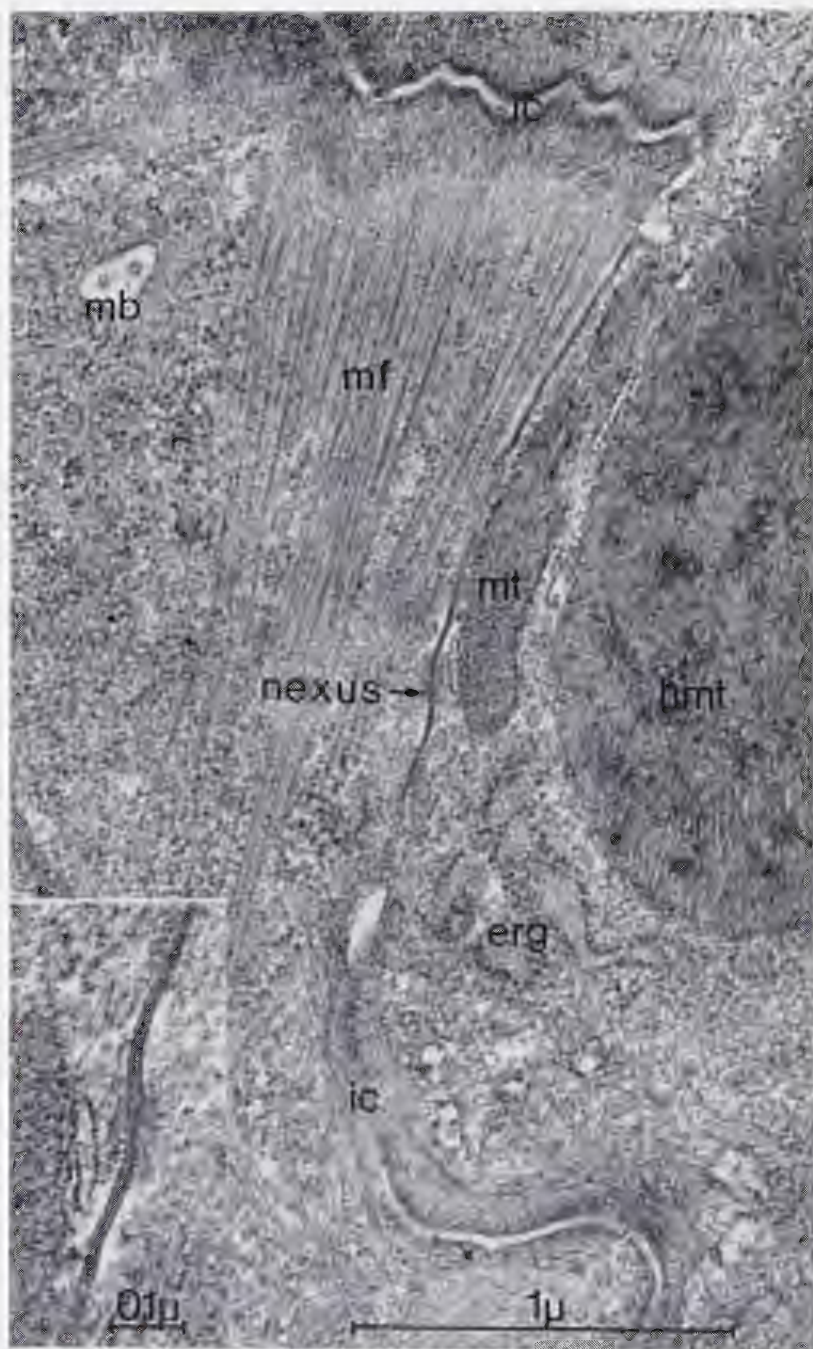


Fig. 5. Junction between two myoblasts, 4 days after culturing. Intercalated disc (ic) with a nexus showing the characteristic quintuple layered structure (see inset) with a thickness of 180 Å. A myofibril in the making (mf) is visible in the upper part of the disc, with dense Z band material adjoining the still denser material of the desmosome; mb, multivesicular body; mt, normal mitochondrion; hmt, hypertrophied mitochondrion; erg, ergastoplasm, magnification 50 000 $\times$. Inset: Magnification 100 000 $\times$.

represents $V(r)$ linearly normalized for $I_0 = 10^{-8}$ A. Superimposed on the plots of the experimental points are the theoretical Bessel functions computed for the best-fitting values of λ and nV_0 .

The results pertaining to electrotonic coupling in each case are presented in Table I. The data concerning mixed cultures were obtained from impalements of pairs of myoblasts only. However, impalements of myoblast-fibroblast pairs also showed a considerable degree of electrotonic coupling.

(c) Morphology

(1) M-M junctions

Marked ultrastructural differences exist between M-M junctions on the hand, and M-F and F-F junctions on the other hand. The regions of contact between cultured cardiac myoblasts (Fig. 5) closely resemble the intercalated discs of adult heart tissue. Two distinct specialized junctional structures, *i.e.* desmosomes and nexuses, are readily recognizable. The nexuses exhibit the characteristic five-layered structure, and their minimal overall thickness is consistently less than 180 Å with our fixation technique. These observations suggest that, structurally, the nexuses connecting cultured cardiac myoblasts are essentially the same as those of heart muscle *in situ*. In the latter tissue, after block-staining with uranyl acetate, and provided the section is adequately oriented with respect to the electron beam, the minimal overall thickness of nexuses is also consistently below 280 Å, and a 20-Å wide gap can regularly be resolved between the outer leaflets of the apposed unit membranes. Using a technique involving impregnation of blocks of myocardial tissue with colloidal lanthanum nitrate, Revel and Karnovsky (1967) were able to demonstrate that the nexal gap possesses a substructure which shows up as a periodic cross-striation in transverse sections and as an hexagonal lattice in tangential sections. The electrical implications of this observation are obvious, since an empty gap, even only 20-Å wide, would act as a shunt for current flowing through the nexus, whilst a closed honeycomb structure would not, or at least not to the same extent.

(2) M-F and F-F junctions

M-F junctions (Fig. 6) exhibit desmosomes, with their characteristic 100-Å wide intercellular cleft and their appositions of electron-dense material on either side of the cleft. However, in contrast to M-M junctions, they are apparently completely devoid of nexuses. They do present points of close contact, at which no intercellular gap is seen. The overall thickness of the merging membranes never falls below the value of twice the thickness of a unit membrane.

In our preparations, no desmosomes were seen at F-F junctions (Figs. 7 and 8). The only specialization encountered at these junctions consisted of focal contacts of the type described above for M-F junctions.

Examination of 10 such points of focal contact, at F-F junctions, was carried out with the aid of a goniometer. Tilting the sections through the appropriate angle showed a definite intercellular cleft in all of them, revealing that the 10 points of

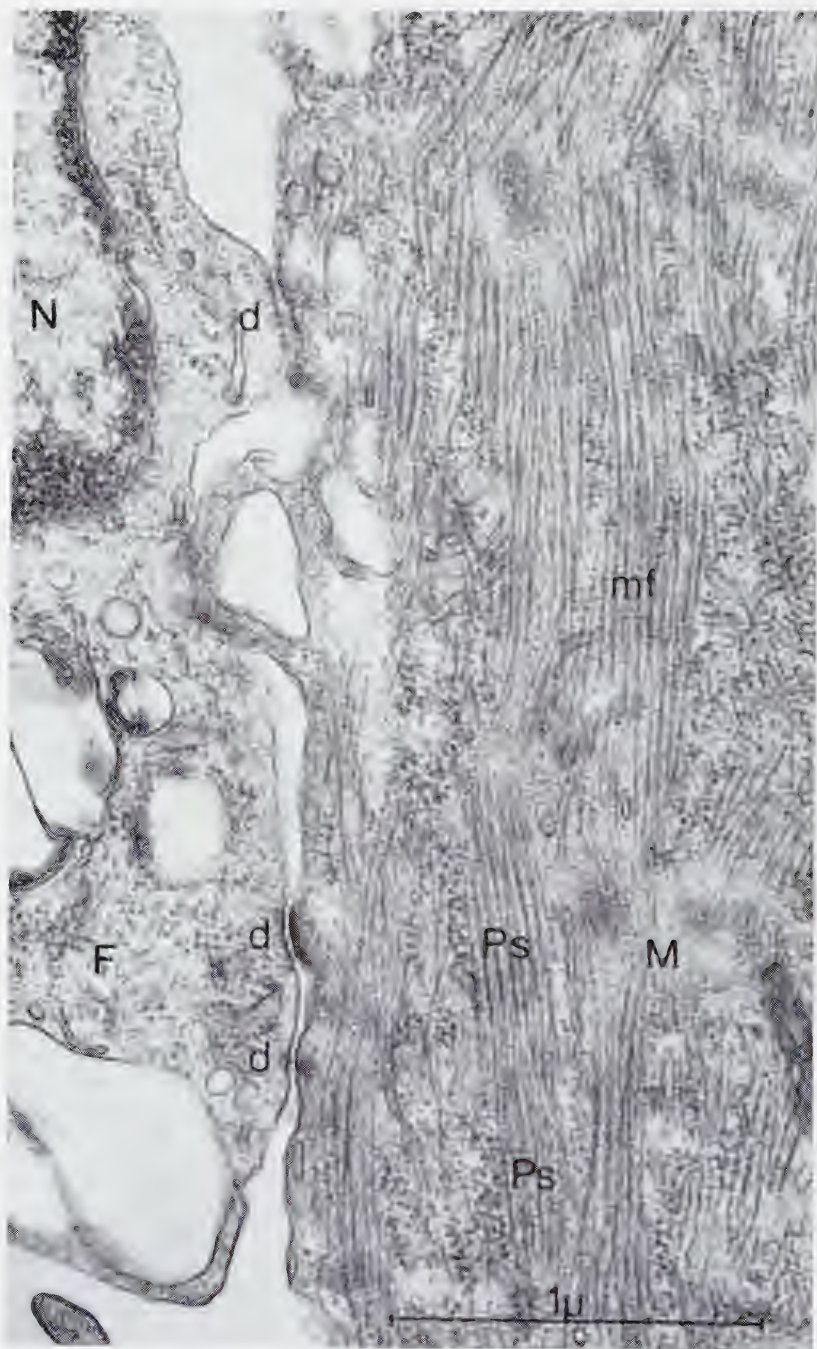


Fig. 6. Myoblast-fibroblast (M-F) junction in a 4-day-old culture, exhibiting a desmosome (d), but no nexus. N, nucleus; Ps, polysomes; mf, myofibrils. Magnification 49 000 $\times$.

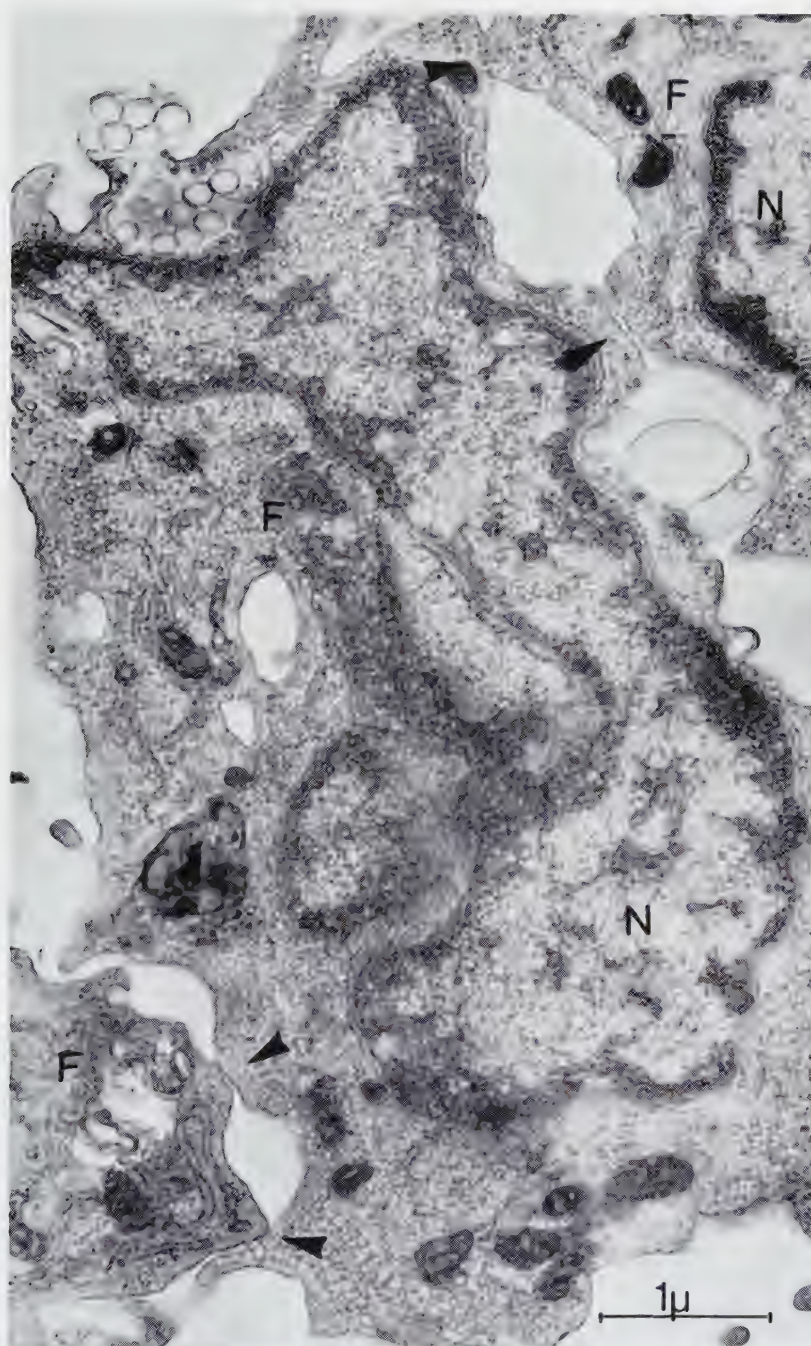


Fig. 7. Three fibroblasts (F) in contact with each other, in a 4-day-old culture. The arrows indicate the points of closest proximity. N, nucleus. Magnification 23 000 $\times$.

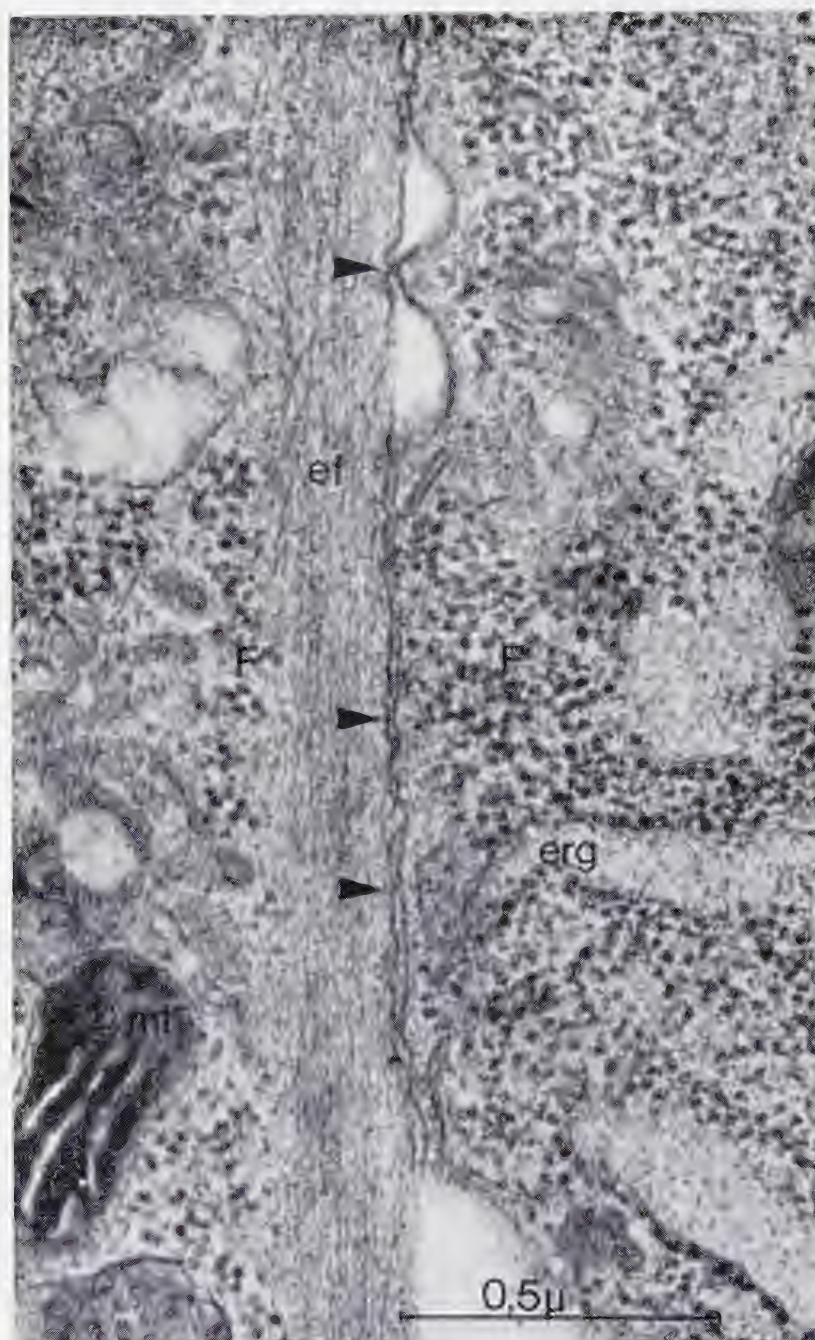


Fig. 8. Fibroblast-fibroblast (F-F) junction in a 4-day-old culture. There are small regions at which the outer leaflets of the apposed membranes apparently touch each other (arrows) but no nexuses; mt, mitochondrion in condensed form (due to hyperosmotic fixative); erg, ergastoplasm; ef, elementary fibrils. Magnification 85 000 $\times$.

contact subjected to goniometric analysis were, in reality, merely superposition artifacts. Sometimes the cleft material was almost electron-transparent, whereas in others it was markedly electron-dense. In Fig. 9, a point of apparent contact is visible at tilt angles between -30 and -6° ; however, tilting the preparation through 0° to $+24^\circ$ reveals a cleft between the two apposed unit membranes. The cleft material is of very low electron-density. Fig. 10 shows apparent fusion of the membranes at tilt angles from -24 to -18° , then increasing separation of the membranes from -12 to $+24^\circ$, followed again by apparent fusion at $+30^\circ$. Even at tilt angles revealing definite separation, the cleft is filled with markedly electron-dense material. Thus the morphological substrate of electrotonic coupling through junctions of this type remains an open question.

DISCUSSION

A survey of recent literature shows that a wide variety of tissues exhibit electrotonic coupling of cells (for references, see Loewenstein, 1966; Bennett, 1968), and one is entitled to ask whether electrical interaction between cells of the same type is not the rule rather than the exception. The purpose of this investigation was to compare the electrotonic properties of two distinct kinds of intercellular junction, formed under identical culturing conditions, and to examine the possibility of metabolic interaction between cells of different types connected with each other through low-resistance junctions.

The results obtained with "pure" cultures of cardiac myoblasts and fibroblasts show that marked differences in the properties of the junctional membranes and plasma membranes existed between these two cell types, as well as differences in their resting potentials. These differences tended to disappear when cells of both types were intimately associated in mixed cultures.

(a) Intercellular junctions

Fig. 4 shows that in "pure" myoblast cultures the plot of the electrotonic potential against the interelectrode distance is remarkably smooth, and that the scatter is small. If the cultures were made of strings of cells in single file, and if high-resistance intercellular junctions be assumed, one might theoretically expect each displacement of the recording electrode from one cell to the next to show up as a definite step in the potential *versus* distance plot. However, this is not so in our preparation because, firstly, a culture is a relatively vast, continuous sheet of cells, and secondly because the curves are statistical. Therefore, instead of showing up as sharp discontinuities in the curves, high-resistance junctions would be reflected in the scatter (background noise) of the points. The experiments reported here show that the scatter was small in "pure" myoblast cultures, slightly larger in mixed cultures, and largest in "pure" fibroblast cultures, indicating that the resistance of F-F junctions is much larger than that of M-M junctions. This suggestion is further supported by a comparison of the calculated values of the overall specific resistance of the intracellular phase

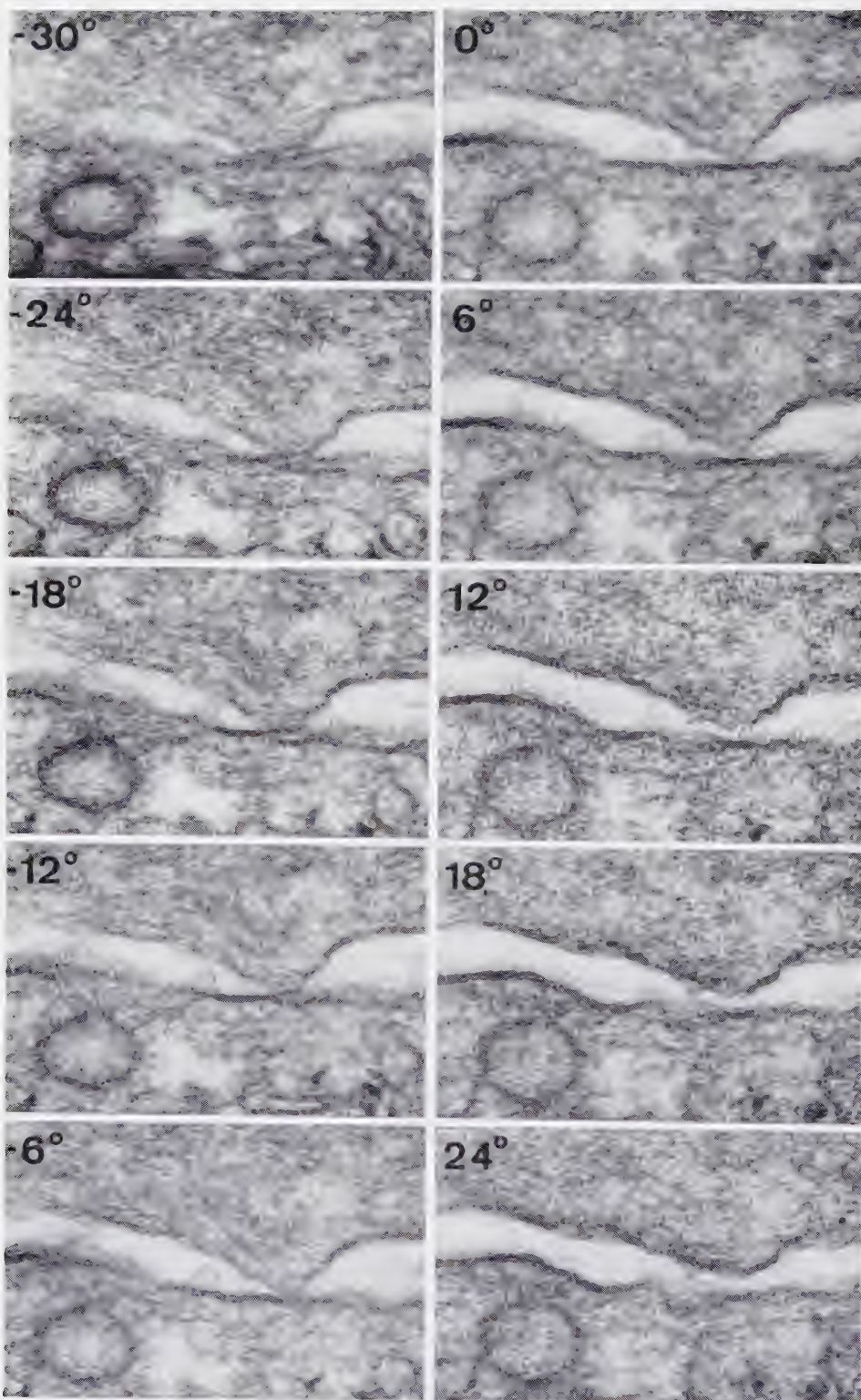


Fig. 9. Goniometric analysis of a junction between two cultured fibroblasts. The numbers refer to tilt angles. All micrographs were selected from a through-focus series. A point of apparent contact is visible at tilt angles between -30 and -6° . Tilting the section to $+24^\circ$ reveals a cleft between the apposed unit membranes. Magnification $150\,000\times$.

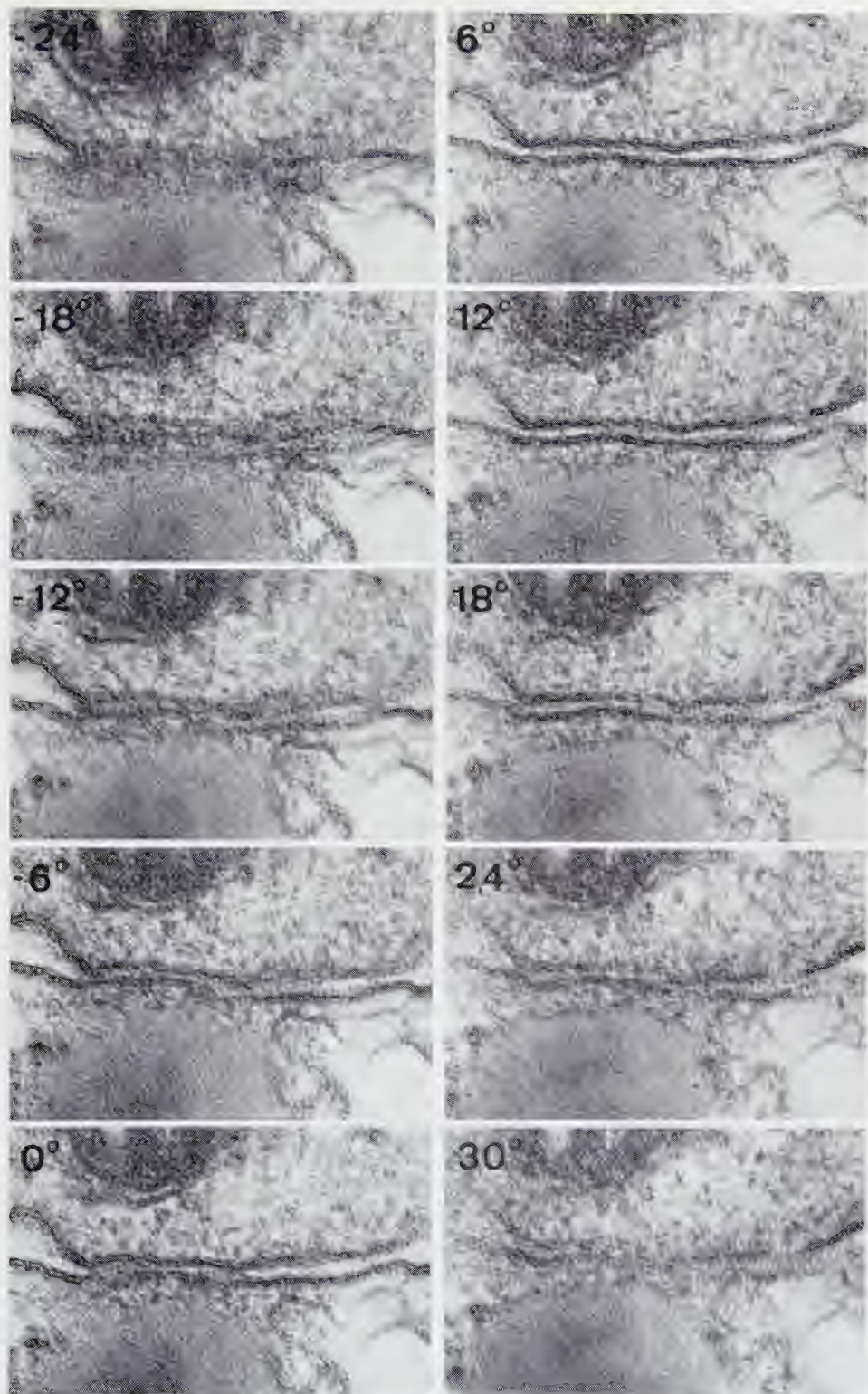


Fig. 10. Same as Fig. 9, except for high electron-density of cleft-material. Magnification 150 000 $\times$.

(R_{i2} , Table I) for both types of pure culture, and for mixed cultures. The mean value of R_{i2} was 419 Ωcm in "pure" myoblast cultures, 604 Ωcm in mixed cultures, and 4134 Ωcm in "pure" fibroblast cultures. It seems highly probable that most, if not all, of the difference between myoblast and fibroblast cultures in this respect is due to the lower effective resistance of the junctional structures between myoblasts.

The mean value of the overall specific resistance of the intracellular medium for cultured myoblasts (419 Ωcm) is 4 times greater than that reported for kid Purkinje fibres (Weidmann, 1952) and 3 times greater than that of calf and sheep Purkinje fibres (Coraboeuf and Weidmann, 1954). Since precise measurements of the nexal area have not been made so far for any of these cell systems, it is impossible to decide whether the specific junctional resistance is higher in cultured myoblasts than in Purkinje fibres, or whether the latter simply provide a larger area of nexus for the flow of current. However, examination of a large number of junctions between Purkinje cells and between cultured myoblasts in the electron microscope leads to a marked preference for the second hypothesis.

The absence of rectification by the intercellular junctions in "pure" cultures of both types, and in mixed cultures, has frequently been checked, since in each double impalement the current was passed alternately through both electrodes. No significant difference in the amplitude of the electrotonic potential was found. The absence of rectification is further illustrated by Fig. 2. Concerning this figure, it may be of interest to mention that a cell could often be depolarized by 30 mV or more without producing an action potential. Anodal break responses, on the other hand, were easily obtained.

Experiments involving injection of minute amounts of tracer substances into a cell and the observation of diffusion of the tracer into neighbouring cells have shown that even quite large molecules, such as fluorescein-labelled serum albumin, could cross at least some types of homocellular junction (Loewenstein, 1966). Diffusion of ions and molecules from one cell to another may be thought to play a part in cell-to-cell transmission of information, as for example in ensuring equivalent differentiation of cells of a given tissue subserving a given set of functions (Dulbecco, 1963). It may reasonably be expected that tight junctions between cells of different types (heterocellular junctions), no matter how tight they might be electrically, would show greater discrimination in the selection of molecules and ions that would be allowed to diffuse through the junctions. The experiments reported in this paper have shown that the resting potential of fibroblasts in mixed cultures is about 3 times higher than that of fibroblasts in pure cultures, suggesting that at least K^+ ions, and presumably also other small ions, must be able to diffuse freely across heterocellular junctions, and that the pumping of ions must be shared. In view of the considerable importance of the intracellular K^+ concentration in the control of many cellular enzymes, in particular those involved in phosphorylation (Kernan, 1965), one can easily imagine that the metabolism of fibroblasts might be profoundly altered by their association with myoblasts.

Another interesting fact concerning M-F junctions, as opposed to M-M junctions, is that in the former we have never seen the characteristic morphology of the nexus.

This observation suggests that formation of a nexus requires that the cells in contact be of the same kind, and may provide some information as to the mechanisms underlying cell-to-cell adhesion. The problem of the precise chemical nature of cell adhesion is still unsolved. However, various hypotheses have been put forward, and can be divided into two major categories: (a) hypotheses involving strictly physico-chemical phenomena, and (b) hypotheses invoking specific cellular functions. Hypotheses of the first category include: stabilization of an intercellular cement by divalent cations (Ringer, 1890; Chambers and Chambers, 1961); calcium-bridge bonding of cell surfaces (Steinberg, 1958; Ambrose, 1967); cell-to-cell adsorption due to the interplay of attractive and repulsive physical forces involving the zeta potentials of the cells; and the London–Van der Waals forces (for detailed discussion of this point, see Beumer *et al.*, 1957). Among the hypotheses of the second category, one can cite bonding between sterically complementary surface groups (Tyler, 1947; Weiss, 1947) and specific cell products acting on the cell surface (Moscona, 1962). The fact that nexuses do not occur at M–F and F–F junctions suggests that their formation requires some kind of specific affinity between the cells involved in the junction, and that the factors governing the formation of nexuses belong to the second category. On the other hand, tight junctions consisting of focal contacts of the outer leaflets of both unit membranes do not seem to require any kind of specific affinity, since they occur both at heterocellular (M–F) and homocellular (F–F) junctions. Further investigations in this field should include a comparative study of the behaviour of both types of junction under various chemical and physico-chemical conditions. Close contacts between cultured fibroblasts, obtained from adult guinea-pigs, have been described by Devis and James (1964). Some of the electron micrographs published by these authors show five-layered junctional structures which most probably represent nexuses.

(b) Plasma membrane

There is a significant negative correlation between the calculated values of R_l and the measured thicknesses of the cultures. This correlation is reasonably well described by a regression line within the limits of the observed range of thicknesses, *i.e.* from 1 to 6 μ , and the equation of the regression line for myoblast cultures is $R_l = 16788 - 2376 d$, where R_l has the dimensions of Ωcm^2 and d is expressed in μ . The explanation of this correlation is found in the geometry of the cell system. Consider Fig. 11, which shows 3 transverse sections through three different myoblast cultures. It can be seen that thickening of the cultures is due to an increase in the number of cell layers, rather than to thickening of the cells. The same holds for fibroblast cultures. If it be remembered that R_l is the leakage resistance of a cm^2 of culture, it becomes obvious that, all other factors being equal, R_l must decrease as the culture becomes more stratified, *i.e.* as the area of cell membrane per cm^2 of culture increases. It is equally obvious that R_l will tend to equal the specific membrane resistance in a strictly monolayer culture. Table I shows that for the thinnest cultures the value of R_l is between 15 000 and 20 000 Ωcm^2 for myoblasts, and approximately 3000 Ωcm^2 for

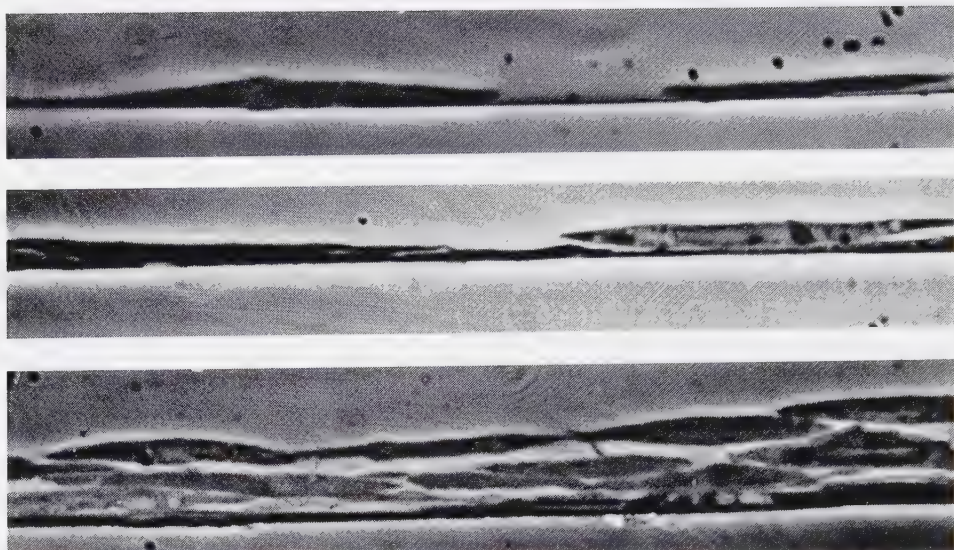


Fig. 11. Transverse sections through 3 myoblast cultures. The increase in thickness is due to stratification of the culture rather than to thickening of the cells.

fibroblasts. These values provide a fairly likely estimate of the specific membrane resistance of both cell types, and show that the ionic membrane permeabilities of myoblasts and fibroblasts are markedly different. That other differences, of a qualitative as well as quantitative nature, also exist between the membranes of the two cell types is suggested by the observation that, under identical culturing conditions, the fibroblasts became attached to the Petri dishes within less than 3 h, whereas the myoblasts required a much longer period.

In a preliminary investigation (Girardier *et al.*, 1967), an attempt was made to determine the specific membrane resistance of cultured myoblasts with a direct method. Completely isolated myoblasts belonging to very young, non-confluent cultures (24–48 h after trypsin dispersion), were impaled with a microelectrode mounted in a Wheatstone bridge, and their effective resistance was measured. The cells were then photographed, and their surface area was estimated with a planimeter. This method yielded a value of the specific membrane resistance of approximately $60 \Omega \text{cm}^2$. Two possible explanations can be put forward for the discrepancy between this result and the results of more recent work. In the first place trypsinization of the cells may cause the membrane resistance to collapse and the latter may take more than 48 h to recover. Secondly, the resting potential of completely isolated myoblasts is regularly low, around 30–40 mV, and it has not been possible to determine whether this is due to a completely isolated cell normally possessing a resting potential of this order, or if it is simply due to damage inflicted by the microelectrode. The second hypothesis seems the more likely, since the resting potential of isolated myoblasts never remained stable for more than a few minutes. If damage to the cell membrane were the cause of the low resting potential, then one would expect a considerable

leakage of current to occur around the electrode tip, and the consequence of this state of affairs would naturally be a low effective resistance.

(c) *Myoblast-fibroblast interactions*

The ionic permeability of the fibroblast plasma membrane is much higher than that of the myoblast plasma membrane. The resting potential of fibroblasts in pure cultures is markedly smaller than that of myoblasts in pure cultures. Further investigation is needed to determine whether the difference in resting potential is due solely to the membrane-permeability factor, or to a difference in the mechanisms of ionic transport through the membranes as well.

The most striking finding is that the difference in magnitude of the resting potential between myoblasts and fibroblasts disappears when both cells are cultured together (mixed cultures). This seems to indicate that intimate association of heterotypic cells causes profound modifications in their membrane properties and/or metabolism, which have not as yet been analyzed experimentally. However, a purely qualitative observation, which may be of interest in this connection, is that satisfactory impalements of fibroblasts were much easier to obtain in mixed cultures than in pure cultures.

Electrotonic coupling between heterotypic cells may be a purely artificial phenomenon, due to the culturing procedure itself, and the pooling of solutes, which it entails, may play a part in the de-differentiation of cells in culture.

(d) *Electrotonic spread*

The results presented in this paper show that the average attenuation length, λ , is approximately 5 times larger in myoblast cultures than in fibroblast cultures. This would seem to indicate that electrical coupling through M-M junctions, whose function is to provide optimal conditions for cell-to-cell flow of current, is from the outset more efficient than through M-F and F-F junctions. Furthermore, it is tempting to speculate that the electrical connections between myocardial cells and cells of the Purkinje fibres are merely remnants of the electrotonic junctions which are known to exist between embryonic cells (Potter *et al.*, 1966). However, this does not seem to be the case, and the impression prevails that tight electrical coupling of myocardial cells is an intrinsic property of these cells, resulting from early specialization of their regions of contact, and of their plasma membrane as a whole.

A further interesting finding was that the greater efficiency of electrical coupling between myoblasts, as compared with that of M-F and F-F junctions, is for a sizeable proportion due to the greater leakage resistance (R_l) of the myoblasts, and not simply to a lower junctional resistance, as might have been assumed on the basis of morphological findings alone. The mean value of the effective resistance of the intracellular medium (r_i) is approximately 7 times as great in fibroblast cultures as in myoblast cultures. Were the leakage resistances (R_l) of myoblast and fibroblast cultures equal, this would account for the space constant of myoblast cultures being only $\sqrt{7}$, or 2.45 times greater than that of fibroblast cultures. On the other hand, the mean

leakage resistance of myoblast cultures is between 3.5 and 5.7 times greater than that of fibroblasts. Division of this factor by the ratio of myoblast effective internal resistance to fibroblast effective internal resistance reasonably accounts for the observed ratio of space constants.

A high specific membrane resistance would seem to be a most useful and economical property for a cardiac myoblast, since it would enable the cell to maintain a sufficiently high resting potential to keep its sodium system in readiness for repetitive action at a relatively low energy cost, and at the same time ensure favourable conditions for the electrotonic spread of excitation.

APPENDIX

(a) Derivation of the basic equations

The model can be schematically represented as in Fig. 12. Let d be the thickness of the sheet of cells, R_i the overall specific resistance of the intracellular medium (myoplasm and intercellular junctions in series), and I_0 the current delivered by one of the intracellular microelectrodes. I_0 divides into I_i , the internal current flowing radially

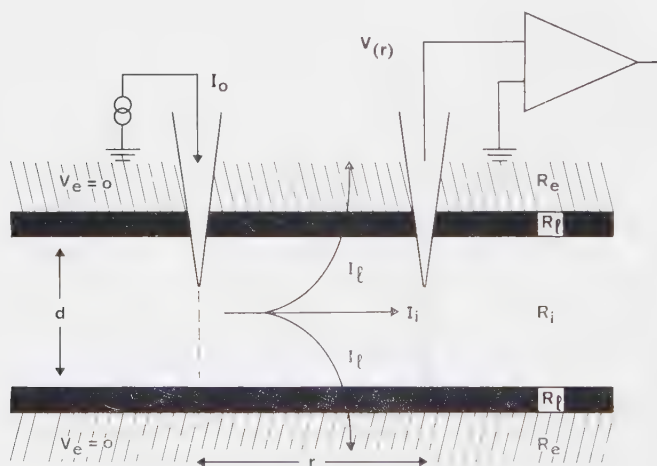


Fig. 12. Diagrammatic representation of the model.

within the myoplasm of the cells, and I_l , the current which escapes through the leakage resistance R_l . If we assume that there is no angular variation of I_i and I_l around the tip of the current electrode, at any finite distance r from the point of application of I_0 the internal current I_i produces a voltage drop $-\Delta V$ across a length Δr of internal medium:

$$-\Delta V = \frac{I}{2\pi r d} \cdot R_i \cdot \Delta r \quad (1)$$

Let
$$\frac{R_i}{d} = r_i \quad (2)$$

then
$$-\Delta V = \frac{I}{2\pi r} \cdot r_i \cdot \Delta r \quad (3)$$

In the differential form, (3) can be written:

$$-\frac{dV}{dr} = \frac{I}{2\pi r} \cdot r_i \quad (4)$$

Differentiating (4):

$$\frac{dI}{dr} = -\frac{2\pi}{r_i} \cdot \left(r \frac{d^2V}{dr^2} + \frac{dV}{dr} \right) \quad (5)$$

Furthermore, the density of current leaking outwards through the upper and lower rings of membrane of width Δr is related to the membrane potential V by the equation

$$-V = \frac{\Delta I}{4\pi r \Delta r} \cdot R_l \quad (6)$$

Photomicrographs of transverse sections through the cultures (Fig. 11) show that the lower aspect of the cells is entirely in contact with the bottom of the Petri dish, but nothing is known about the precise nature of the contact nor of its electrical resistance. Hence some uncertainty exists as to the leakage area of the bottom layer of cells. In the differential form, (6) becomes

$$\frac{dI}{dr} = -\frac{4\pi}{R_l} \cdot r \cdot V \quad (7)$$

If we assume the specific resistance of the extracellular fluid to be negligible and combine eqns. (5) and (7):

$$-\frac{4\pi}{R_l} \cdot r \cdot V = -\frac{2\pi}{r_i} \left(r \cdot \frac{d^2V}{dr^2} + \frac{dV}{dr} \right) \quad (8)$$

Let
$$\frac{R_l}{r_i} = \lambda^2 \quad (9)$$

then
$$\frac{d^2 V}{dr^2} + \frac{1}{r} \cdot \frac{dV}{dr} - \frac{2}{\lambda^2} \cdot V = 0 \quad (10)$$

A particular solution of eqn. (10) meeting our boundary conditions for steady state is

$$V_{(r)} = nV_o K_o(x) \quad (11)$$

where $V_{(r)}$ = deflection of membrane potential due to current pulse I_o at distance r from the current electrode

n = constant depending on the boundary conditions

$V_o = V$ at finite distance r_o from point source of current

$K_o(x) = (\pi/2) i H_o^{(1)}(ix)$, *i.e.* modified zero order Bessel function of second kind with imaginary argument

$$x = \frac{r}{\lambda}$$

If one turns back to eqn. (7), one can see that the total amount of current which leaks out of the cells between the tips of both electrodes is

$$I_{l(r)} = - \frac{4\pi}{R_l} \cdot \int_{r_o}^r V_{(r)} \cdot dr \quad (12)$$

Replacing $V_{(r)}$ by its value in (10)

$$I_{l(r)} = - \frac{4\pi}{R_l} \cdot nV_o \cdot \int_{r_o}^r r K_o(x) dr \quad (13)$$

The internal current at distance r is

$$I_{i(r)} = I_o - I_{l(r)}$$

Eqn. (4) can now be re-written

$$- \frac{dV}{dr} = \frac{r_i}{2\pi r} (I_o - I_{l(r)}) \quad (14)$$

Since

$$\frac{r_i}{R_l} = \frac{1}{\lambda^2}$$

and letting

$$\int_{r_o}^r r K_o(x) dr = \varphi$$

eqn. (14) becomes

$$- \frac{dV_{(r)}}{dr} = \frac{I_o r_i}{2\pi r} - \frac{2nV_o \varphi}{\lambda^2 r} \quad (15)$$

The practical integration formula for φ is

$$\varphi = \sum_{r=r_o}^{r=r} \left[\frac{K_o(x)}{2} (r_2^2 - r_1^2) \right] \quad (16)$$

(b) Curve fitting

The experimental results provide two of the terms contained in eqn. (11), namely $V_{(r)}$ and r . The remaining terms, *i.e.* nV_0 and λ , were determined by curve fitting as follows. The measured values of $V_{(r)}$ for a given culture were linearly normalized for $I_0 = 10^{-8}$ A, and plotted semilogarithmically against r . Justification for linear normalization will be found in Fig. 2. The regression line of the voltage-distance plot was drawn according to the least-square method. The plot was then compared with families of Bessel curves made out beforehand for various values of λ and nV_0 , until a suitable degree of coincidence was obtained. The fit was considered satisfactory when the regression line of the experimental points coincided with that of the points of the theoretical Bessel curve comprised within the limits of r tested in the experiment. The values of

$$K_0(x) = \frac{\pi}{2} i H_0^{(1)}(ix)$$

were taken from tables of functions (Jahnke and Emde, 1938). Term R_i was calculated using eqn. (15), in which

$$-\frac{dV_{(r)}}{dr}$$

was measured graphically and φ was integrated in steps of $\Delta x = 0.04$, starting arbitrarily at $x_0 = 0.02$ in all cultures. The values of R_i and R_l were computed from eqns. (2) and (9) respectively.

SUMMARY

(1) A method for preparing practically pure cultures of cardiac myoblasts and fibroblasts is described. With this technique it has been possible to investigate the properties of three functionally distinct types of cell junction, and the effects of association of heterotypic cells.

(2) Under identical culturing conditions, the attenuation length of pure myoblast cultures was found to be 5 times greater than that of pure fibroblast cultures. This difference is due both to the lower overall specific resistance of the intracellular medium of myoblast cultures, and to the higher specific membrane resistance of these cells. The attenuation length of mixed cultures was intermediate between the two extremes.

(3) Electrotonic coupling was found between heterotypic cells (myoblasts and fibroblasts).

(4) The measured membrane potential of fibroblasts was 3 times higher when they were in intimate association with myoblasts in mixed cultures than in pure cultures. Conversely, the resting potential of stray myoblast contaminants in predominantly fibroblastic cultures was of the same magnitude as that of the surrounding fibroblasts.

(5) Neither myoblast-myoblast junctions, nor fibroblast-fibroblast junctions exhibited rectification.

(6) Typical nexuses were found at myoblast-myoblast junctions, but not at fibroblast-fibroblast and myoblast-fibroblast junctions. This suggests that electrotonic coupling may occur through junctional structures other than the nexus.

(7) At fibroblast-fibroblast and myoblast-fibroblast junctions the appearance of the points of closest proximity between the adjoining cells was suggestive of focal contact of the outer leaflets of the unit membranes. Even rotation of the section under the electron beam by means of a goniometer did not entirely clarify the precise nature of these focal contacts.

(8) The results at hand suggest that formation of nexuses at a cell junction requires that the partners in the junction be homotypic.

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